Single-molecule studies of membrane transporters

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1 | Introduction

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Cells are delineated by a lipid bilayer that physically separates and protects the inside from the outer environment. Although some small molecules can pass this membrane, most polar, charged or large molecules require proteins for transport at rates that are relevant for life. Here I introduce the basic properties of the biological membrane and key aspects of a number of protein transporters, especially those that belong to the ATP-binding cassette (ABC) superfamily. I also present a short overview of the single-molecule fluorescence techniques used in this thesis to study membrane proteins in their native environment.

Membrane transport

Cells are the fundamental units of life, both structurally and functionally[1]. Alone or organized in multicellular organisms they exhibit great diversity. Despite this variation, all cells share similar biological molecules they use to support life and propagate. Molecules ranging from small nutrients to large proteins and nucleic acids make up the interior of a cell, which is enclosed by a membrane. In eukaryotic systems, cellular organelles exist within the cell with each their own membrane. In this thesis, I will exclusively focus on bacterial systems, with the membrane acting as a physical interface between the inside and outside environments. The cell membrane comprises a lipid bilayer with a thickness of approximated 5 nm in which proteins are embedded. The membrane bilayer is a highly dynamic and fluid structure and it is formed spontaneously as the hydrophobic tails of the lipids organized themselves to prevent unfavourable interactions with the aqueous environment (Fig. 1.1a)[2]. Although the (protein-free) lipid bilayer is impermeable to macromolecules, some small molecules can, depending on their size and electrical charge, diffusive through the bilayer along a gradient (Fig. 1.1a)[3]. Small hydrophobic molecules such as O_2 and CO_2 can dissolve in the lipid bilayer, and thus diffuse across. Uncharged polar molecules, for example H_2O, glycerol, and the larger glucose, will also diffuse through, though at a (much) lower rate. Charged molecules, such as protons[†] and other ions, are unable to pass the hydrophobic core of the bilayer due to their hydration.

In order to precisely control intracellular solute concentrations and to allow transport of molecules that are otherwise unable to cross a lipid bilayer, cells employ dedicated, membrane-integrated transport proteins. The high portion of genes encoding for transport proteins reflects their importance: 20-30% of genes encode for membrane proteins[4], half of which are transporters[5]. Transport proteins are divided in two main classes: passive and active transporters (Fig. 1.1b)[6]. Passive transporters utilize the energy of the electrochemical gradient of the substrate molecules to move the solute in the direction of the gradient across the membrane. They can be divided in two classes: (i) channels or pores that form a physical opening, which can be controlled, as a selective filter to a specific molecule, and (ii) carrier proteins that have a binding site for a specific molecule and undergo conformational changes

[†] In practice, protons may actually cross the membrane through (transient) defects[3].

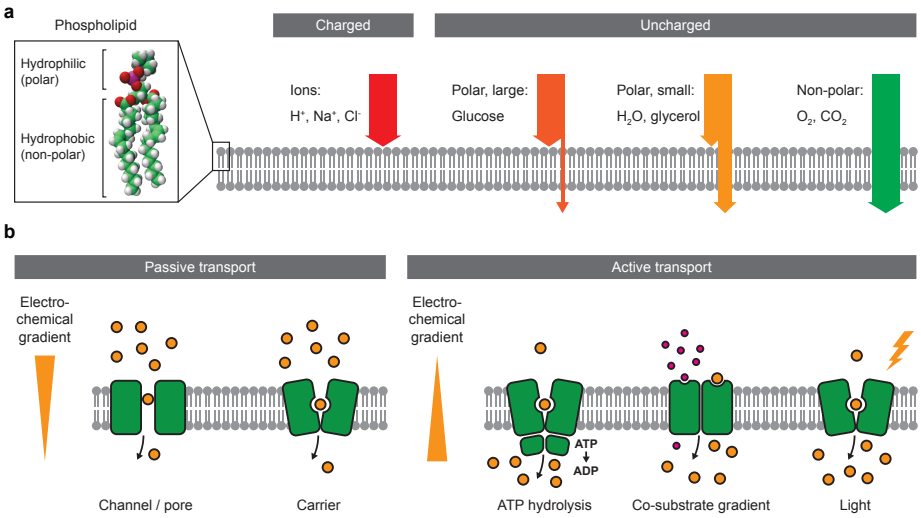

Figure 1.1 | **Membrane transport. a** Phospholipids (depicted in box) spontaneously form a lipid bilayer in aqueous solution. The bilayer is permeable to small uncharged molecules; the permeability depends on polarity and size of the molecule. Charged molecules are unable to diffuse through the membrane. **b** Transport proteins can transport solutes that otherwise would not be able to pass the membrane. Passive transporters (channels/pores and mediators) use the energy of the electrochemical gradient of the substrate. Active transporters use other sources of energy to transport a substrate against a gradient: chemical energy from ATP hydrolysis, the energy of a co-substrate gradient, or other sources such as light.

such that only one side of the membrane is accessible to that molecule. Active transporters use different sources of energy to transport a specific molecule or class of molecules against a gradient. Primary active transporters use energy provided by ATP hydrolysis to drive conformational changes of the protein. Secondary (coupled) transporters use the electrochemical gradient of a co-substrate (for example Na^+) to transport their main substrate. Other active transporters employ light or redox to drive the transporter. Transport of larger molecules, such as proteins, or even larger particles typically requires a different transport mechanism and is beyond the scope of this thesis.

ABC Transporters

ATP-binding cassette (ABC) transporters form the largest superfamily of active transport proteins. They can be found in organisms ranging from prokaryotes to humans and they perform a multitude of tasks[7]. Despite their variety, all ABC transporters share the same architecture with two nucleotide binding domains (NBDs) forming the core of the complex together with two transmembrane domains (TMDs) (Fig. 1.2). In a number of classes of ABC transporters, a soluble substrate-binding protein (SBP), acting as a receptor for the substrate, complements the system. The NBDs are highly conserved throughout all ABC transporters. Binding and hydrolysis of ATP at the interface between these subunits drive conformational changes of the TMDs, and thereby mediate transport of substrate across the membrane. The TMDs have evolved from multiple ancestors[8]. Their (diverse) structure classifies the protein complexes and determines their substrate specificity[9]. To date, five types of

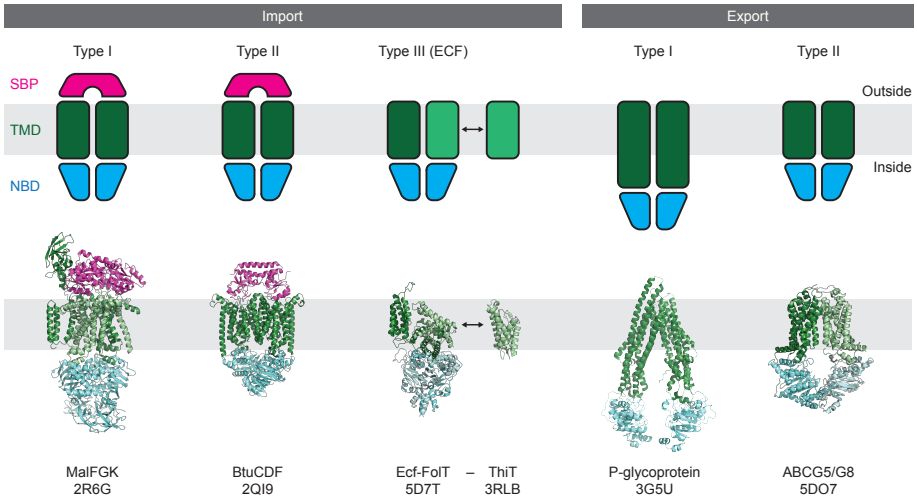

Figure 1.2 | ABC transporters. Three types of ABC importers and two types of ABC exporters can be distinguished based on their TMD structure (top row). They all share the same general design: two highly conserved NDBs (blue) drive conformational changes in the TMDs (green) to allow transport of substrate. Type I and II importers are complemented with an SBP (magenta); type III (ECF) transporters are characterized by an exchangeable substrate-specific TMD subunit. The bottom row shows crystal structures of well-studied model cases. Protein Data Bank (PDB) identifiers are depicted below the names.

transporters have been identified (Fig. 1.2). Three types of *importers*, only found in prokaryotes, can be distinguished based on the TMD structure: type I, II and III (the latter also called energy coupling factor (ECF) transporters). Their distinct structures correspond to different transport mechanisms[10–16]. ABC *importers* are mostly responsible for the import of small cellular nutrients such as sugars, amino acids, metal chelates and vitamins. ABC *exporters* are involved in the transport of hydrophobic compounds such as lipids, cholesterol, drugs and even larger molecules as proteins. They play a fundamental role in multidrug resistance[17], cancer[18] and human diseases[19] like cystic fibrosis[20]. Two types of *exporters* with different transmembrane folds are currently known[21].

While structural and biochemical studies have provided tremendous insights into ABC transporters, many mechanistic questions remain unanswered[22]. For a large number of transporters it is unknown how many ATP molecules are hydrolyzed per translocated substrate molecule, and which step in the ATP hydrolysis cycle provides the necessary conformational changes. There is also uncertainty about the association state of the different subunits (TMDs, NBDs and SBPs) throughout the transport cycle. Therefore, high-resolution structural data have to be complemented by biochemical and advanced biophysical studies that provide dynamic insight.

Membrane proteins and their environment

Membrane proteins are naturally embedded in a lipid bilayer, providing a complex and dynamic environment. In many experimental approaches, the proteins have to be removed from their native surroundings before they can be analyzed further in a detergent or lipid environment *in vitro*[23]. Like lipids, detergents are amphiphilic molecules with a polar (potentially charged) head and a hydrophobic tail. They can solubilize membrane proteins by covering the bilayer-embedded elements, thus resembling the natural lipid bilayer (Fig. 1.3)[24]. Compared to such detergent-based solubilization, a lipid bilayer reconstituted *in vitro* resembles the native environment much more closely. Detergent-solubilized membrane proteins can be reconstituted in nanodiscs[25] or liposomes[26] of various sizes (Fig. 1.3). Nanodiscs are small disc-shaped patches of membrane surrounded by scaffolding proteins acting as a belt; the diameter of around ten nanometres can be controlled by the length of the scaffolding proteins that define the circumference of the disc. Embedded integral membrane

proteins are experimentally accessible from both sides. Liposomes, however, limit the accessibility to the outside – the inside can be pre-loaded with the desired constituents, though. Their diameters vary from below 100 nm to tens of micrometres; unilaminar liposomes are typically in the lower size range. The compartmentalization offered by liposomes allows the establishment of gradients and makes them of great interest for studying transport.

Mimicking the complex and dynamic native environment of transport proteins is nontrivial. Biological membranes consist of multiple components that determine properties such as thickness, strength and tension[23]. At the same time, membrane proteins interact with the surrounding lipids[27,28], affecting protein folding and functioning[29]. Carefully choosing the lipids used for protein reconstitution minimizes any negative effects. Prokaryotic transporters are typically reconstituted in a mixture of dioleoyl-phosphatidyl-ethanolamine (DOPE), dioleoyl-phosphatidylglycerol (DOPG) and dioleoyl-phosphatidylcholine (DOPC), as they require a significant fraction of non-bilayer (i.e. cone-shaped, as opposed to cylinder-shaped) lipids (DOPE) and anionic lipids (DOPG)[30]. For eukaryotic systems, typically a sterol is included as well.

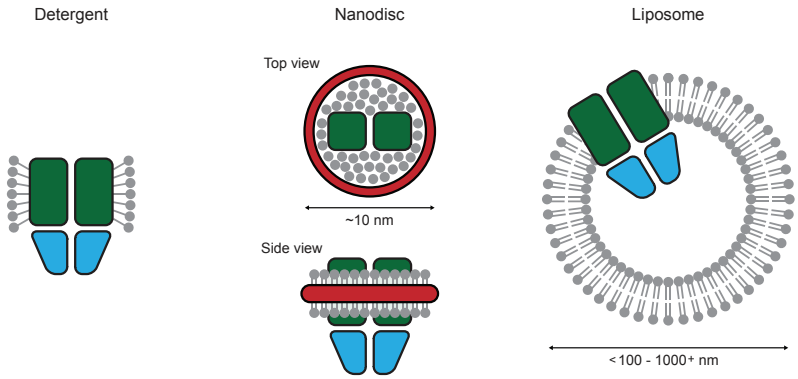

Figure 1.3 | Environment of membrane proteins. In an aqueous environment, membrane proteins can be stabilized in detergent (left). Here the hydrophobic tails of the detergent molecules cover the protein and mimic the bilayer. Nanodiscs (middle) and liposomes (right) resemble the native environment most closely. In nanodiscs, a scaffolding protein (red) holds a patch of lipids together while the membrane protein is accessible from either side. Liposomes on the other hand allow for gradients and allow only the outside to be accessible during the measurement, which is beneficial for studying transport.

Experimental methods: single-molecule techniques

Extensive structural and biochemical investigations already have provided great understanding of membrane transporters. These techniques, however, provide ensemble-averaged readouts and conceal many aspects of the dynamics of individual members of the unsynchronized population. Single-molecule techniques overcome this limit by directly probing individual proteins and thereby providing new insights into important structural and functional properties. The first single-molecule observations were made in the 1970s by the detection of currents in single ion channels[31]. Advances such as the detection of single fluorescent molecules under biologically relevant conditions[32] and single fluorescent proteins[33], as well as the continuous improvement of high-sensitivity and low-noise photon detectors made single-molecule techniques a powerful tool to study membrane proteins *in vitro* and *in vivo*. Although other methods such as atomic force microscopy (AFM) have been established to probe the dynamics of single membrane proteins[34], in this thesis I rely on fluorescence techniques that will be described in more detail below.[†]

Fluorescence is the radiative decay, in the form of light, from a molecular singlet state back into the ground state from which the molecule has been excited[35]. The wavelength of the emitted light is typically higher than the wavelength of the light used for excitation; a phenomenon called Stokes shift. Fluorophores – or dyes – are specifically designed molecules with optimized fluorescence properties such as stability, brightness and pH sensitivity. When chemically linked to a protein (or other molecule) they can be used to investigate the host in several ways; a few of which will be described below (Fig. 1.4a)[36]. First of all, they can be utilized in fluorescence microscopy to determine the spatial position with an accuracy of a few tens of nanometre. Although the point source of light produces a spot (point spread function – PSF) with a diameter that is fundamentally limited by the wavelength of the light, the centre can be localized much more accurately[37]. When two host molecules are labelled with fluorophores with different spectral properties (i.e. different colours), they can be co-localized. Second, intensity fluctuations of a single dye can report on the activity or dynamics of proteins[38]. The host can change the local environment of the dye and thereby quench or enhance fluorescence. Lastly, fluorescence (or Förster) resonance energy transfer (FRET) is a mechanism based on the non-radiative transfer

[†]Numerous other single-molecule detection and manipulation techniques exist to probe a great variety of complex biological processes, but they are beyond the scope of this thesis.

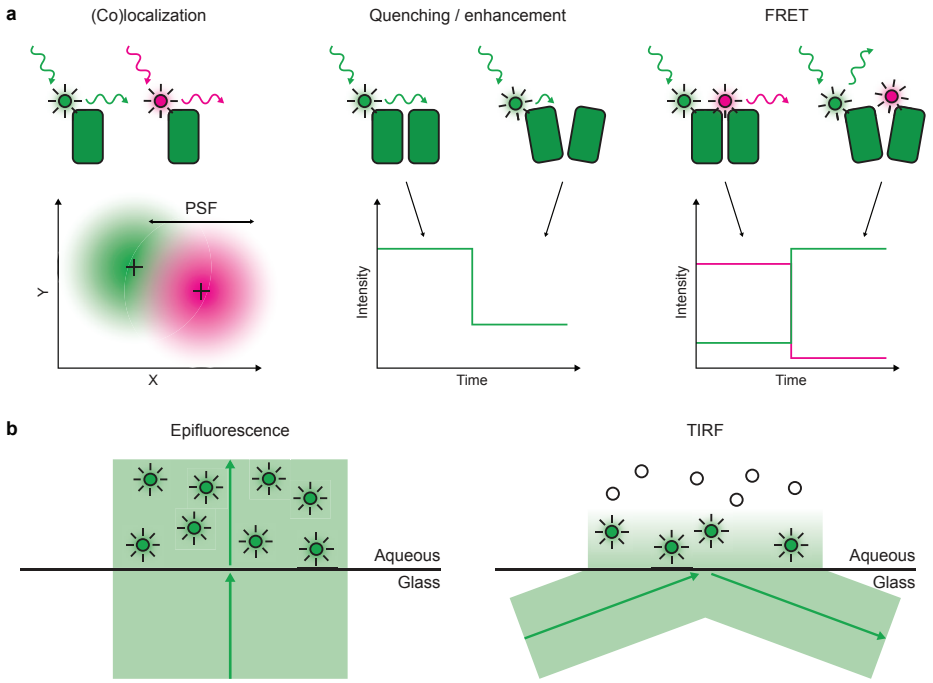

Figure 1.4 | Single-molecule techniques. **a** Several fluorescence techniques; principle (top) and observable (bottom). By fitting the PSF (typically a few 100 nm wide), the fluorophore can be localized (crosshairs) with an accuracy of a few 10 nm (left). Using multiple colours can hint for colocalization. Changes in local environment, which can depend on protein conformation, of the fluorophore can cause quenching or enhancement (middle). FRET can be used to measure small distance changes between roughly 20 and 100 Å (right). **b** In epifluorescence (left), the entire aqueous solution is excited by the light beam, whereas in TIRF (right) only a shallow layer (~100 - 200 nm) on top of the glass surface is excited.

of excitation energy from a donor to an acceptor fluorophore[39]. This transfer results in a decreased fluorescence intensity of the donor, and an increased intensity of the acceptor. The efficiency E of the transfer depends on the distance R between donor and acceptor:

$$E = \frac{1}{1 + (R/R_0)^6}.$$

Here R_0 is the Förster distance at which the efficiency is 50%. The Förster distance depends on the pair of dyes and is typically between 50 and 80 Å. When both dyes are placed accordingly, it allows the detection of the interaction between molecules or even structural changes within a biomolecule on the length scale of a few nanometres.

Although chemical and photophysical properties of fluorophores are constantly being improved[40], they do have a limited rate and number of photons emitted. These limitations result in a requirement for efficient and low-background detection of emission light. The first need is realized by high-quality optics and high-sensitivity single-photon detectors. Minimizing the amount of background photons, mainly originating from scattering and impurities, can be done in two ways. In confocal imaging the detection volume is minimized to volumes on the order of femtolitres[41], thereby typically allowing only one molecule to be measured. The molecule can be either freely diffusing or immobilized on a surface. A point detector captures all photons originating from the detection volume. An alternative method to reduce background photons, which we also employ here, is to decrease the excitation volume by using total internal reflection fluorescence (TIRF) microscopy[42]. As opposed to epifluorescence, where the excitation light beam crosses through the entire sample, TIRF relies on an incident excitation light beam that is totally reflected inside a glass coverslip, thereby creating an evanescent wave of approximately 100 to 200 nm thick (Fig. 1.4b). As a result, only fluorophores that are close to the surface, typically those whose host is immobilized on the surface, will be excited. Emitted photons are projected on a 2-dimensional camera, which enables the recording of multiple fluorophores in parallel.

Thesis outline

In this thesis, I mainly focus on single-molecule studies of membrane transporters in order to answer outstanding questions about their mechanisms that biochemical studies have not been able to answer so far. Here, I provide a brief outline of its various chapters, together with a short introduction of the studied subjects.

Glutamate transporters are a family of neurotransmitter transporters that are responsible for uptake of the excitatory neurotransmitter glutamate from the synaptic cleft[43]. The proteins in this family, which also includes aspartate transporters found in prokaryotes, are secondary active transporters. The sodium-coupled aspartate transporter Glt_{Ph} from *Pyrococcus horikoshii* is a well-studied archaeal member of the family. The transporter is a homotrimer; each protomer has a transport and a scaffolding domain and uses an elevator-like alternating-access mechanism[44–48]. One aspartate molecule is co-transported with three sodium ions[49,50]. The positions of all sodium binding sites have been identified with X-ray crystallography[46,51,52]. Furthermore, simulations revealed possible extracellular and intracellular gating mechanisms for substrate binding and release[53,54]. Although the homotrimeric protein has been studied extensively, the dynamic behaviour of the individual subunits and their coordination is unknown. By employing single-molecule FRET imaging and TIRF microscopy we have studied the motion of individual subunits of liposome-reconstituted Glt_{Ph} (Chapter 2).

The vitamin B_{12} importer BtuCD-F in *Escherichia coli* (*E. coli*) is a very well-studied type II ABC importer (see Appendix I: Brief background on vitamin B_{12}). Several states of the transporter have been captured in crystal structures[12,13,55–57], and the gating mechanism has been studied by EPR spectroscopy[58,59]. The transporter has also been the subject of computer simulations, which showed the coupling between the movements of the NBDs and TMDs[60,61]. Additionally, the importer has been investigated in liposomes and detergent with ensemble biochemical studies[62,63]. The numerous structural and biochemical investigations have led to varying transport models, but several steps of the transport mechanism are still not completely understood. Here, we have probed single transporters in liposomes with various single-molecule fluorescence techniques and TIRF microscopy to visualize the transport of a single vitamin B_{12} molecule, while undergoing conformational changes (Chapter 3).

Imaging of fluorescent proteins in single bacterial cells has shown that proteins localize to specific subregions[64]. Although membrane proteins always colocalize with the cell perimeter, other proteins might show varying spatiotemporal behaviour[65]. When the abundance of proteins of interest is low, information from many cells needs to be combined to make the protein location visible. Commonly, cell segmentation techniques are employed, with software packages such as MicrobeTracker[66], CellProfiler[67,68], Schnitzcells[69], PSICIC[70], BactImAS[71], and Oufti[72]. Although these packages are flexible, they have disadvantages: the algorithms typically require extensive tuning and they are computationally expensive. In this work we investigated a new and fast user-impartial technique to overlay cells along the short axis (Chapter 4).

Appendix

I. Brief background on vitamin B_{12}

Vitamin B_{12}, also known as cobalamin, is a water-soluble co-factor essential for many cellular processes. Discovered as a cure for pernicious anaemia, later also called vitamin B_{12} deficiency anaemia, in 1948[73], the structure was only solved 8 years after[74]. Many organisms, ranging from bacteria to eukaryotes, need the vitamin, however a few bacteria can synthesize it themselves[75,76]. The classical model organism *E. coli* can not synthesize the vitamin *de novo*.

The structure of cobalamin is based on a corrin ring surrounding a cobalt ion (Fig. 1.A1a). The metal ion is coordinated by six sites: four of them are on the corrin ring, one is the lower ligand dimethylbenzimidazole (DMBI) and one is a variable upper ligand. This centre of reactivity can be a cyano group, a hydroxyl group, a methyl group or a 5'-deoxyadenosyl group. Cyanocobalamin† is the stable synthetic form that is biologically inactive, but can be metabolized into one of the two active forms methylcobalamin or adenosylcobalamin. These biologically relevant forms are unstable as the lower ligands can experience photo-induced dissociation. All four forms of cobalamin have a strong absorption peak around 350 nm (Fig. 1.A1b).

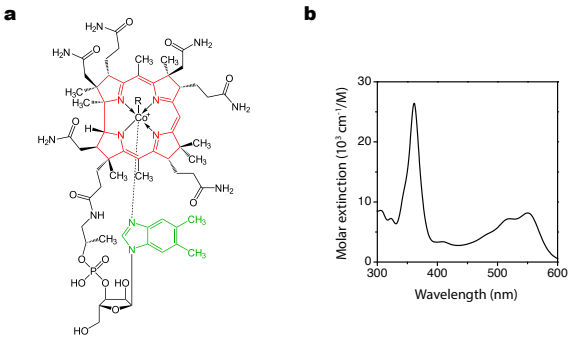

Figure 1.A1 | Structure and absorption spectrum of cobalamin. a Structure of cobalamin. The cobalt ion is coordinated by a corrin ring (red), a lower ligand DMBI (green) and a variable upper ligand (R). **b** Absorption spectrum of cyanocobalamin in 50 mM KPi, 200 mM KCl, pH 7.5.

†When not specified further, by convention cyanocobalamin is the form of vitamin B_{12} used.

To date, the only vitamin B_{12} uptake system known in bacteria is BtuCD-F, which resides in the inner membrane of *E. coli*. Cobalamin, having a molar mass of 1355 g/mol, is unable to diffusive passively through the outer membrane of the gram-negative bacterium. Therefore this scarce nutrient is actively transported into the periplasm by the BtuB transporter interacting with the Ton-system which delivers the required energy[77]. Subsequently, vitamin B_{12} is scavenged from the periplasm and imported into the cytosol by BtuCD-F.

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